A peer-reviewed open-access journal
NeoBiota
Advancing research on alien species and biological invasions

NeoBiota 95: 279–290 (2024)
DOI: 10.3897/neobiota.95.124673

Research Article

# Reliable molecular detection of small hive beetles

Orlando Yañez[1], Marga van Gent-Pelzer[2], Anna Granato[3], Marc Oliver Schäfer[4], Peter Neumann[1]

1 *Institute of Bee Health, University of Bern, CH-3097 Bern, Switzerland*
2 *Wageningen University & Research, Biointeractions & Plant Health, P.O. Box 16, 6700 AA Wageningen, Netherlands*
3 *Istituto Zooprofilattico Sperimentale delle Venezie, National Reference Laboratory for Honey Bee Health, Viale dell'Università 10, 35020 Legnaro (PD), Italy*
4 *Friedrich-Loeffler-Institut - Federal Research Institute for Animal Health, Greifswald - Insel Riems, 17493, Germany*
*Corresponding author: Orlando Yañez (orlando.yanez@unibe.ch)*

## Abstract

Invasive species require adequate reliable detection methods to mitigate their further spread and impact. However, the reliability of molecular detection methods is often hampered by both false positives (Error type I) and false negatives (Error type II). At present, the reliability of the four published molecular detection methods for small hive beetles (SHB), *Aethina tumida*, has not been rigorously evaluated considering their extensive genetic diversity. Here, we performed intra- and interlaboratory comparisons of the four available methods using SHB samples representing 78 regions from 27 countries on five continents, beetles from the same genus (*Aethina concolor*, *A. inconspicua*, *A. flavicollis* and *A. major*), as well as western honey bees, *Apis mellifera*, and ectoparasitic mites *Varroa destructor*. The data show that the Idrissou et al. (2018) and Li et al. (2018) methods avoid both false positives and false negatives probably due to lower sensitivity to nucleotide mismatches on the primer and probe's target sequences. Further, the Li et al. (2018) method can be considered more sensitive because the fluorescent amplification curve crosses the threshold at lower Cq values compared to the Idrissou et al. (2018) one. In light of our data, the Li et al. (2018) method is the most reliable molecular diagnostic tool for SHB. We therefore recommend using this method as it will contribute to management efforts of this invasive species.

**Key words:** *Aethina tumida*, inter-laboratory comparison, qPCR, ring test

OPEN ACCESS

**Academic editor:** Victoria Lantschner
**Received:** 3 April 2024
**Accepted:** 10 September 2024
**Published:** 15 October 2024

**Citation:** Yañez O, van Gent-Pelzer M, Granato A, Schäfer MO, Neumann P (2024) Reliable molecular detection of small hive beetles. NeoBiota 95: 279–290. https://doi.org/10.3897/neobiota.95.124673

**Copyright:** © Orlando Yañez et al.
This is an open access article distributed under terms of the Creative Commons Attribution License (Attribution 4.0 International – CC BY 4.0).

## Introduction

The small hive beetle (SHB), *Aethina tumida*, is a parasite and scavenger of honey bee colonies that is continuing to invade the world since it was first noticed outside its natural distribution, in Africa, south of the Sahara, in 1996 in the USA (Hood 2000). As infestation of honey bee colonies with *A. tumida* did cause severe damage to apiculture in all the new areas where *A. tumida* has been introduced to (Neumann and Elzen 2004; Ellis and Hepburn 2006), it has been added to the lists of notifiable diseases of the World Organization for Animal Health (WOAH) and the European Union (EU). However, despite comprehensive elimination and contingency efforts, it already has established local populations on every continent except Antarctica and it is likely to continue spreading (Neumann et al. 2016; Schäfer et al. 2019).

A reliable method for the early detection of SHB specimens in places where they are not endemic provides the opportunity to have a cost-effective management of the situation which will look to prevent the initial establishment of SHB, and therefore, minimize the ecological and economical effects of this invasive species. However,

despite this obvious advantage, the reliability of the different DNA-based detection methods for SHB is unknown. The molecular methods based on the detection of SHB's DNA have the advantage of identifying not just the adults but the insect's early developmental stages as well. Indeed, the taxonomical identification of early stages as eggs is a difficult task if it is based only on the morphology. However, a potential limitation of most molecular methods for the detection of SHB is that they were designed with limited information of SHB's DNA variability. Actually, the primers (and probes) from most methods were designed with DNA information from specimens collected in introduced areas and few specimens from the African continent where the beetle is widely distributed and where the higher genetic diversity is expected. Therefore, it is quite important to test the reliability of the molecular methods using a much larger number of SHB specimens from their continent of origin (Idrissou et al. 2019).

Over the last 15 years several PCR methods had been developed for the detection of SHB. This study compares four genetic detection methods to evaluate their effectiveness, sensitivity and specificity, identifying the strengths and limitations of each method, aiming to identify the most accurate one. Three of those diagnostic methods were designed for using the hydrolysis probe technique (Ward et al. 2007; Li et al. 2018; Silacci et al. 2018). Those assays include a sequence-specific, oligonucleotide probe labelled with a fluorescent reporter and a quencher of fluorescence at opposite ends, in addition to the sequence-specific PCR primers. The hydrolysis method exploits the 5' to 3' exonuclease activity of the *Taq* polymerase. At the PCR extension step, once the polymerase reaches the probe, its exonuclease activity degrades the probe cleaving off the fluorescent reporter. As a result, it is separated from the quencher, resulting in a fluorescence signal. Probe-based qPCR enables the amplification of more than one target in a single reaction using different reporters with distinct fluorescent spectra. As this technique uses specific primers and probes to the target sequences, it is regarded as a technique with very high specificity. Besides the methods using the hydrolysis probe technique, also a detection method designed for conventional PCR (Idrissou et al. 2018) was evaluated. However, for this study the method was modified to test if those primers were suitable for the SYBR Green qPCR method. The qPCR non-specific detection method uses SYBR Green as fluorescent dye. This dye emits fluorescence when binds to double stranded DNA (dsDNA). Therefore, the fluorescence intensity is proportional with the concentration of dsDNA. It is considered a non-specific method because the dye binds to dsDNA, independent of the nucleotide sequence. The absent of specific-sequence fluorochrome-labelled probe make its use less expensive. However, the specificity relied entirely on the design of the primers to avoid the risk of nonspecific PCR amplifications. Commonly, this is verified assessing the melting temperature (Tm) of the amplicon by melting curve analyses that take place after the qPCR runs.

There are two error types that are of special importance to evaluate, error types I and II. Error type I, also known as a false positive, occurs when a method incorrectly identifies the presence of SHBs when they are absent. Error type II, also known as a false negative, occurs when a method fails to detect the presence of SHBs when they are actually present. For the evaluation of those parameters of the SHB qPCR detection methods, it was particularly necessary to test them using an extended collection of SHB specimens. In this study, the collection has representative specimens from the three known SHB phylogenetically clades (Liu et al. 2021) and were sampled from 4 non-endemic continents and a significant contribution from Africa (44 out of 78 total regions, and 16 out of 27 total countries) since it

is the continent of the species origin holding the major genetic diversity (Idrissou et al. 2019). This is important to highlight as most of that genetic information was not available when the tested detection methods were developed, which implies that some haplotypes may have not been considered during the design of these methods, which may have consequences in their accuracy.

Finally, for further validation of the detection method comparison, we performed a blind ring test and an inter-laboratory comparison test between laboratory partners dedicated to the detection of SHB. For the ring test, the participating laboratories blindly tested selected SHB haplotypes using their own routine methods. For the inter-laboratory comparison test all participating laboratories used the selected most accurate method to tests its reproducibility and sensitivity across these laboratories.

## Materials and methods

### Comparison of methods

The objective of these tests was to establish the capabilities of different proposed qPCR methods to reliably confirm the detection of SHB.

#### Samples

Adult SHB (N = 83) representing 78 regions from 27 countries on five continents from the collection at the Institute of Bee Health (IBH, University of Bern, Switzerland) were selected (Suppl. material 1: table S1). Beetles from the same genus (*Aethina concolor*, *A. inconspicua*, *A. flavicollis* and *A. major*; N = 1 each; Suppl. material 1: table S1) were also selected and used to test the specificity of the methods. Workers of western honey bees *Apis mellifera* (N = 2) and ectoparasitic mites *Varroa destructor* (N = 2) collected in Switzerland were also included. After collection, all beetle samples were preserved in 70% ethanol, transported at room temperature and stored at –80 °C. The DNA extraction (from the whole specimen bodies), DNA yield and purity (using a spectrophotometer) and the Cytochrome Oxidase I gene (COI) barcoding protocols are described in Idrissou et al. (2019).

#### Selection of methods

Four DNA-based published methods were considered (Ward et al. 2007; Idrissou et al. 2018; Silacci et al. 2018 and Li et al. 2018). For internal control, a hydrolysis probe assay targeting a common region of the 18S rRNA gene was used (Silacci et al. 2018). The sets of primers and probes are detailed in Suppl. material 1: table S2. At the IBH, all the specimens detailed in the "Samples" section were tested following the protocols as described by Ward et al. (2007); Silacci et al. (2018) and Li et al. (2018), see Suppl. material 1: table S3. In contrast, the PCR conditions described by Idrissou et al. (2018) were adapted to SYBR Green qPCR conditions (Suppl. material 1: table S3).

### Ring test using routine detection methods

The objective of the ring test was to establish the proficiency of the participant laboratory's routine method for the detection of SHB.

SHB DNA from single specimens among the three major SHB phylogenetic clades (clade A: Italy (Cosenza-Calabria), clade B: Burkina Faso (Bobo Dioulasso) and Tanzania (Arusha), clade C: Philippines (Davao); Liu et al. 2021) were tested blindly by the four participant laboratories. Besides belonging to a particular phylogenetic clade, those samples were selected because their detection status was not uniform among the methods described above. In addition, to test for interspecific cross detection, DNA samples from single individuals from *A. concolor* (Australia), *A. flavicollis* (South Korea) and *A. mellifera* (Switzerland) were also included in this assay (see "Samples" section). Three technical replicates per sample were provided in 20 µl volume per replication. DNA concentration of the delivered samples ranged from 1 to 5 ng/µl. The SHB DNA samples were prepared at the IBH and delivered to the other three laboratories (Table 1) on dry ice for preservation.

The routine method used by each laboratory and the respective amplification conditions are described in Suppl. material 1: table S4.

## Inter-laboratory comparison using the selected detection method

The objective was to establish the proficiency of the Li et al. (2018) SHB probe-based qPCR detection method under laboratory conditions of each participant.

The DNA samples (including the replicates) used in the ring test assay were used as well for the inter-laboratory comparison. In addition, each laboratory was also provided with ten-fold serial SHB DNA dilutions (from $5*10^{-3}$ to $5*10^{-9}$ ng/µl) in order to determine the sensitivity of their qPCR assays for this method. The SHB DNA used for the dilutions belong to a sample from Clade B (Burkina Faso, Bobo-Dioulasso), which was previously shown to be positively detected by the described Li et al. (2018) method. Each dilution was provided with three technical replicates (20 µl volume each). The primers and probes for the COI (Li et al. 2018) and 18S rRNA (Silacci et al. 2018) regions were also provided to each laboratory by the IBH. Both sets of primers and probes were set to work simultaneously in multiplex. The qPCR conditions were performed following Li et al. (2018) (Suppl. material 1: table S3).

**Table 1.** Ring test for the comparison of SHB PCR detection methods. Specimens of *Aethina tumida*, *Aethina flavicollis*, *Aethina concolor* and *Apis mellifera* were screened (blind test) by each participating laboratory using their own routine detection method. Positive detection is expressed by the respective Cq value. No detection (nd).

| Species | Sample location | Phylogenetic clade | Institute of Bee Health (Switzerland) | | | Istituto Zooprofilattico Sperimentale delle Venezie (Italy) | | | Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health (Germany) | | | WUR Biointeractions & Plant Health (The Netherlands) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Li et al. 2018 method | | | Ward et al. 2007 method | | | Ward et al. 2007 method | | | Li et al. 2018 method (LNA modified) | | |
| | | | Rep. 1 | Rep. 2 | Rep. 3 | Rep. 1 | Rep. 2 | Rep. 3 | Rep. 1 | Rep. 2 | Rep. 3 | Rep. 1 | Rep. 2 | Rep. 3 |
| *A. tumida* | Italy (Cosenza-Calabria) | A | 22.99 | 22.21 | 22.74 | 35.32 | 35.38 | 35.25 | 29.29 | 28.34 | 28.21 | 27.63 | 27.44 | 27.54 |
| *A. tumida* | Burkina Faso (Bobo Dioulasso) | B | 17.25 | 17.37 | 17.47 | nd | nd | nd | nd | nd | nd | 20.36 | 20.62 | 20.74 |
| *A. tumida* | Tanzania (Arusha) | B | 20.55 | 20.31 | 20.18 | 21.76 | 22.06 | 22.41 | 22.23 | 21.59 | 22.63 | 24.33 | 24.23 | 24.44 |
| *A. tumida* | Philippines (Davao) | C | 20.11 | 19.62 | 19.92 | 39.41 | nd | nd | 34.21 | 35.1 | 34.04 | 23.48 | 23.63 | 23.42 |
| *A. flavicollis* | South Korea | - | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| *A. concolor* | Australia | - | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| *A. mellifera* | Switzerland | - | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| - | Negative control ($H_2O$) | - | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |

### Statistical analyses

The SHB detection methods were pairwise compared using the Bland-Altman method comparison technique (Altman and Bland 1983) which tests the limits of agreement of two measurements of the same variable. The tests were performed using the NCSS 2022 Data statistical software. It provides the correlation coefficient and a diagnostic test to determine if the differences are normal (Test of normality of differences, Shapiro-Wilk, $\alpha = 0.05$).

## Results

### Comparison of detection methods

All methods were able to discriminate *A. concolor*, *A. inconspicua*, *A. flavicollis*, *A. major*, *A. mellifera* and *V. destructor* from *A. tumida*, implying that false positive results were not detected. In the case of hydrolysis probe methods (Ward et al. 2007; Li et al. 2018; Silacci et al. 2018), no amplification curves were observed above the auto-calculated threshold set by the qPCR software version (Bio-Rad CFX Maestro 1.0 Version 4.0.2325.0418). In the case of the SYBR Green qPCR detection method (Idrissou et al. 2018, modified), as the threshold was crossed by the *A. mellifera* samples (Cq values of 33.34 and 33.95), melting curve analyses were used to discriminate *A. tumida* samples from *A. mellifera*: *A. tumida* peak at Tm of 79.0–79.5 °C whereas *A. mellifera* peak at Tm of 84.0–84.5 °C (Suppl. material 1: fig. S1).

However, the Ward et al. (2007) and the Silacci et al. (2018) methods produced some false negative results (Figs 1, 3, 4). Ward et al. (2007) did not detect beetles from Burkina Faso (Bobo Dioulasso) and Silacci et al. (2018) did not detect specimens collected from Burkina Faso (Bobo Dioulasso, Fada-Ngourma and Tenkodogo), Burundi (Rusiga) and Italy (Cosenza-Calabria). On the other hand, Li et al. (2018) and the modified Idrissou et al. (2018) effectively detected all specimens with no false negative results.

For the pairwise comparison between methods (Fig. 2), the Li et al. (2018) method was chosen as the reference because it showed lower variability of its Cq detection values (Fig. 1). The outliers under the inferior limit of agreement in all three comparisons (Fig. 2a, b, c) show that the Li et al. (2018) method detects the SHB samples at lower Cq values, significantly (Test of normality of differences, Shapiro-Wilk, $p < 0.001$ for all three comparisons).

### Ring test using each participant's routine detection method

SHB DNA from single specimen collected in Italy (Clade A), Burkina Faso (Clade B), Tanzania (Clade B) and Philippines (Clade C) were tested blindly by each participant laboratory. The laboratories that used Ward et al. (2007) method failed to detect the SHB specimen from Burkina Faso, which accumulated 16 mismatches between primers and probe together (Suppl. material 1: fig. S2). In contrast, the laboratories using Li et al. (2018) method and its modified version (probe designed with Locked Nucleic Acid bases) were able to detect all of the tested specimens (Table 1). No interspecific cross detection was shown, neither for any of the used routine methods nor for any of the participant laboratories, on the DNA samples from specimens of *A. concolor*, *A. flavicollis* and *A. mellifera*.

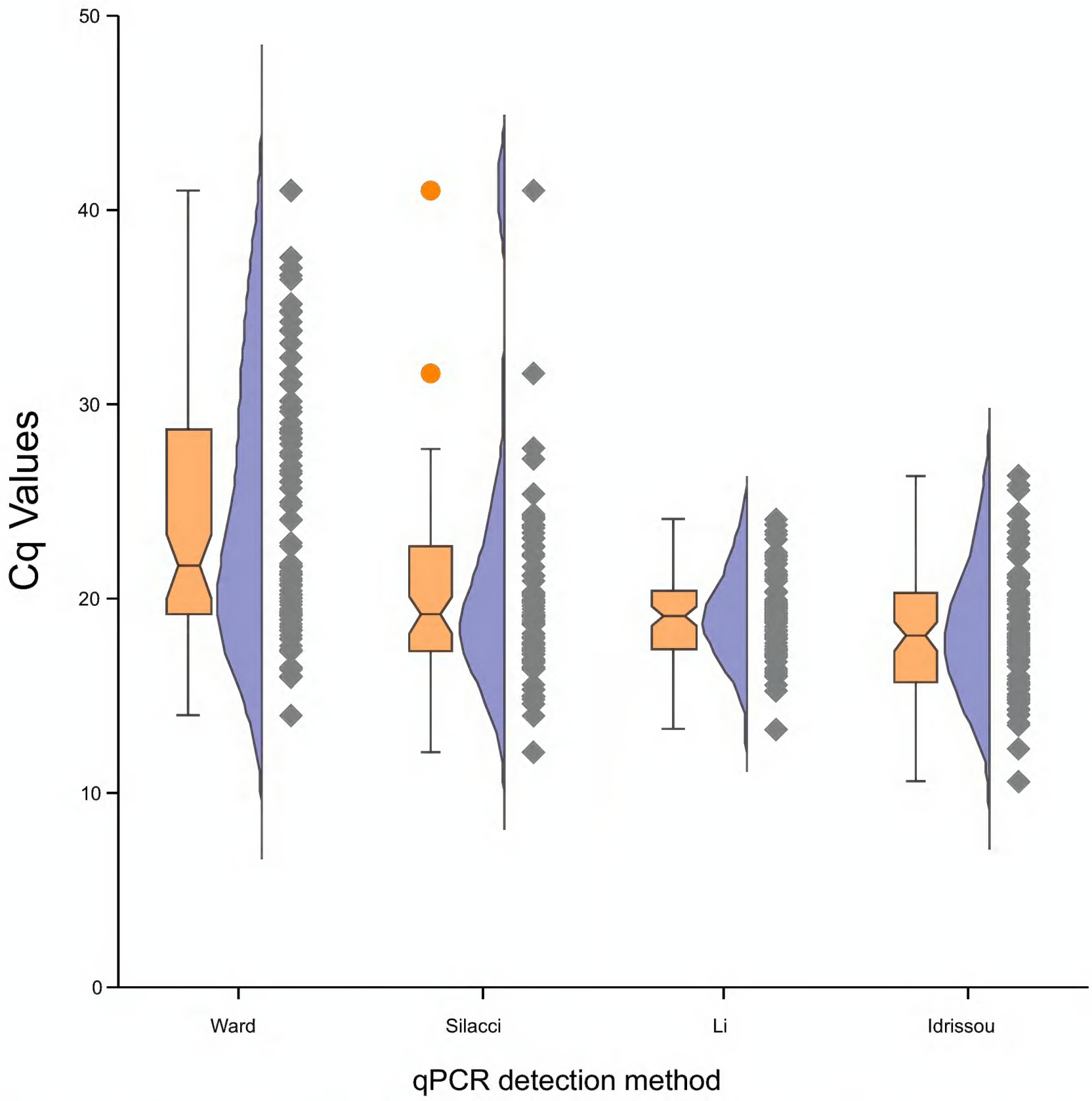


**Figure 1.** Cq value (N = 83) distribution for the different small hive beetle qPCR detection method expressed in box, density and dot plots. A Cq value of 41 was assigned in case of no small hive beetle detection.

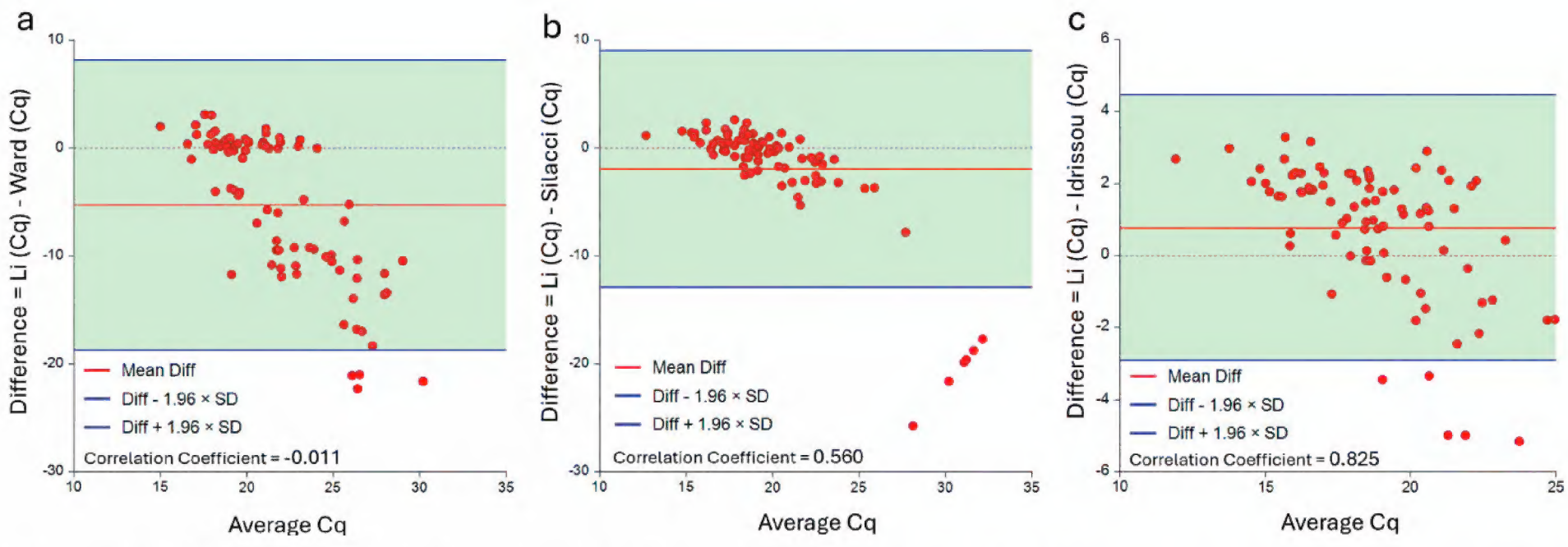


**Figure 2.** Bland-Altman pairwise method comparison. The vertical axis plots the Cq value differences between Li et al. (2018) and **a** Ward et al. (2007) **b** Silacci et al. (2018) and **c** Idrissou et al. (2018). The average Cq of the compared methods is plotted along the horizontal axis. The horizontal red line represents the mean of the differences. The blue horizontal lines define the limits of agreement using the z-value = 1.96 (95% CI).

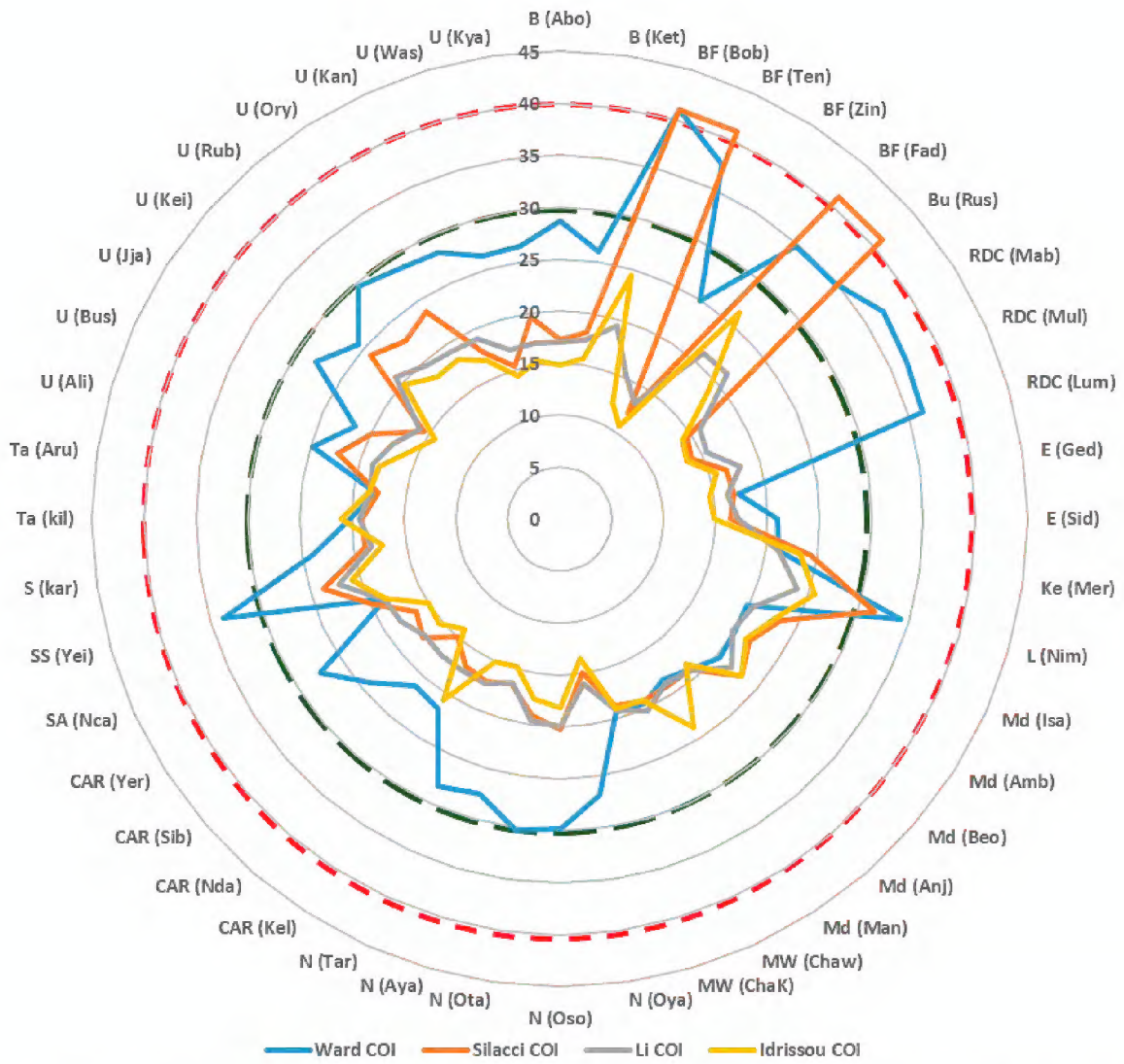


**Figure 3.** Cq value distribution for each different small hive beetle qPCR detection method for specimen from the endemic African range. Red dash line represents Cq value 40, the limit of detection. Green dash line represents Cq value 30. A Cq value of 41 was assigned in case of no SHB detection. B: Benin, BF: Burkina Faso, Bu: Burundi, DRC: Democratic Republic of Congo, E: Ethiopia, Ke: Kenya, L: Liberia, Md: Madagascar, MW: Malawi, N: Nigeria, CAR: Central African Republic, SA: South Africa, SS: South Sudan, S: Sudan, Ta: Tanzania, U: Uganda. Initials of the site of collection in parentheses (i.e., Abo = Abomey).

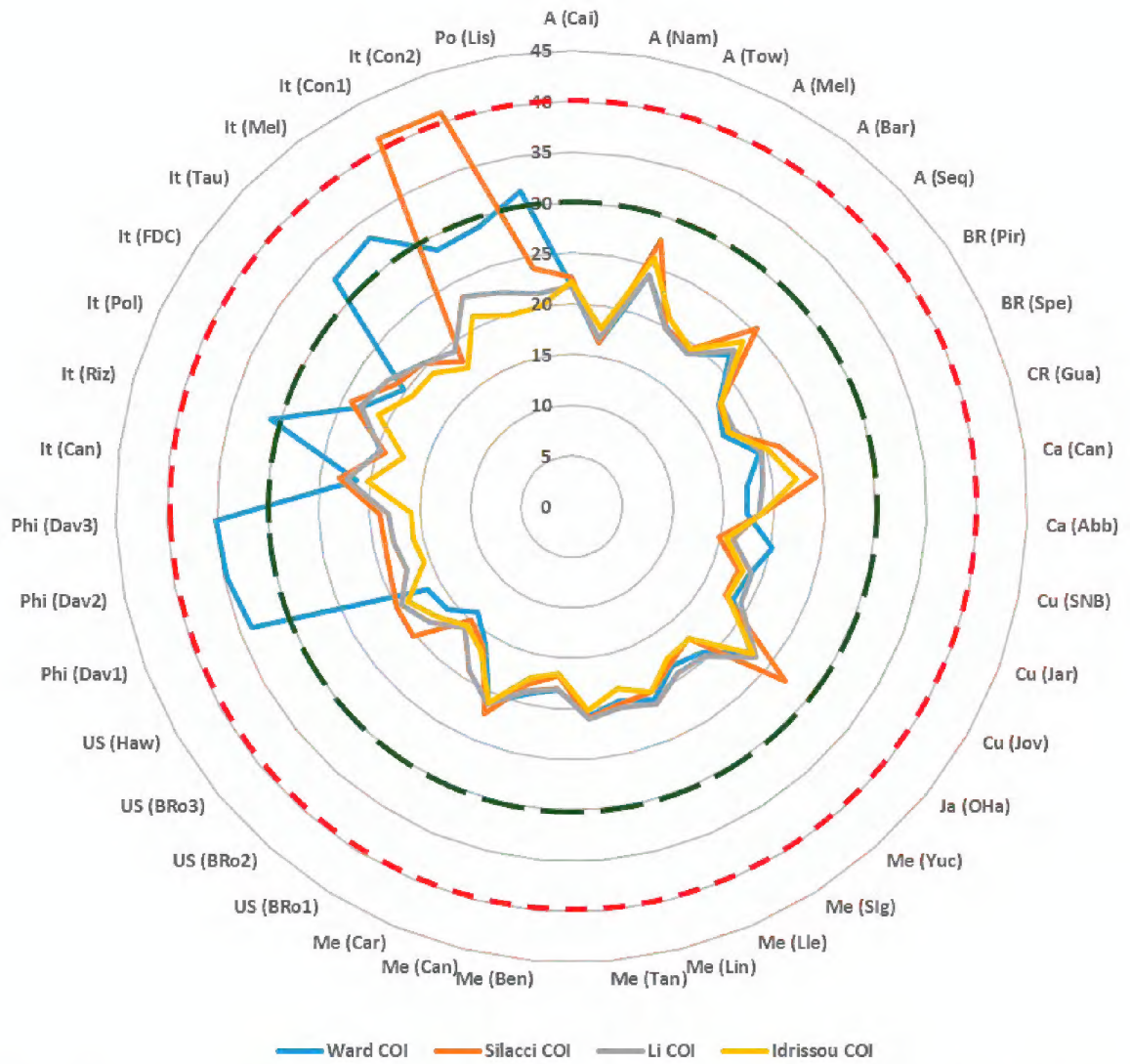


**Figure 4.** Cq value distribution for each small hive beetle qPCR detection method for small hive beetle specimen collected from countries in the invasive range. Red dash line represents Cq value 40, the limit of detection. Green dash line represents Cq value 30. A Cq value of 41 was assigned in case of no SHB detection. A: Australia, BR: Brazil, CR: Costa Rica; Ca: Canada, Cu: Cuba, Ja: Jamaica, Me: Mexico, US: USA, Phi: Philippines, It: Italy; Po: Portugal. Initials of the site of collection in parentheses (i.e., Cai = Cairns).

## Inter-laboratory comparison using the selected SHB detection method

From the results of the "Comparison of detection methods" section, the Li et al. (2018) method was chosen for the inter-laboratory SHB detection method comparison test. The proficiency of this method for the qPCR detection of SHB proved to be robust and reliable under each participating laboratory conditions. After performing the blind test, all of the SHB DNA samples, from different phylogenetic clades, were correctly confirmed by each participating laboratory (Table 2). Similarly to the previous assays, no interspecific cross detection (false positive results) was detected, for any of the DNA samples from specimens of *A. concolor*, *A. flavicollis* and *A. mellifera*.

The proficiency of Silacci et al. (2018) method for the qPCR detection of insect DNA (18S rRNA gene) was also tested to serve as an internal control for DNA quality and/or the presence of PCR inhibitors (e.g., ethanol). Insect DNA was detected in all specimens but not in some replications of the *A. concolor* specimen (Table 3).

Regarding the sensitivity of the Li et al. (2018) method, the highest sensitivity (positive detection of the three replicates with the lower DNA dilution) was detected at $5*10^{-6}$ ng/µl SHB DNA at the time the samples were freshly prepared. After the samples were delivered and tested under each participant laboratory conditions, detection of 100% of the samples for all laboratories was reached at $5*10^{-3}$ ng/µl SHB DNA (Table 4). This include samples that were unplanned and exposed to room temperature storage for 5 days due to delayed custom clearance during sample delivery.

## Discussion

This study compared the effectiveness and specificity of the four DNA-based detection methods for SHB, *A. tumida*, published over the last 15 years. Our data clearly show that Li et al. (2018) and Idrissou et al. (2018) were the only methods that accurately detected all tested samples (N = 83) including 44 regions from Africa. This is the largest diversity of SHB specimens ever tested with those methods. The Idrissou et al. (2018) method was originally designed as an end point PCR method. However, this study proves that this method can be adapted for SYBR qPCR and

**Table 2.** Inter-laboratory comparison test of the Li et al. (2018) method for the PCR detection of SHB. Specimens of *Aethina tumida*, *Aethina flavicollis*, *Aethina concolor* and *Apis mellifera* were screened (blind test) by each participating laboratory. Positive detection is expressed by the respective Cq value. No detection (nd).

| Species | Sample location | Phylogenetic clade | Institute of Bee Health (Switzerland) | | | Istituto Zooprofilattico Sperimentale delle Venezie (Italy) | | | Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health (Germany) | | | WUR Biointeractions & Plant Health (The Netherlands) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Rep. 1 | Rep. 2 | Rep. 3 | Rep. 1 | Rep. 2 | Rep. 3 | Rep. 1 | Rep. 2 | Rep. 3 | Rep. 1 | Rep. 2 | Rep. 3 |
| *A. tumida* | Italy (Cosenza-Calabria) | A | 22.99 | 22.21 | 22.74 | 27.93 | 28.27 | 28 | 32.99 | 32.6 | 29.51 | 27.63 | 27.75 | 27.53 |
| *A. tumida* | Burkina Faso (Bobo Dioulasso) | B | 17.25 | 17.37 | 17.47 | 20.94 | 20.91 | 20 | 26.3 | 25.5 | 23.11 | 19.49 | 20.05 | 19.92 |
| *A. tumida* | Tanzania (Arusha) | B | 20.55 | 20.31 | 20.18 | 25.55 | 25.19 | 25.52 | 26.21 | 27.56 | 29.09 | 24.36 | 24.59 | 24.74 |
| *A. tumida* | Philippines (Davao) | C | 20.11 | 19.62 | 19.92 | 22.56 | 22.05 | 22.23 | 23.86 | 24.47 | 24.02 | 21.45 | 21.37 | 21.31 |
| *A. flavicollis* | South Korea | - | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| *A. concolor* | Australia | - | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| *A. mellifera* | Switzerland | - | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| - | Negative control ($H_2O$) | - | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |

**Table 3.** Inter-laboratory comparison test of the Silacci et al. (2018) method for the PCR detection of the insect's 18S rRNA gene. This method controls for amplifiable insect DNA. Specimens of *Aethina tumida*, *Aethina flavicollis*, *Aethina concolor* and *Apis mellifera* were screened (blind test) in each laboratory. Positive detection is expressed by the respective Cq value. No detection (nd).

| Species | Sample location | Phylogenetic clade | Institute of Bee Health (Switzerland) | | | Istituto Zooprofilattico Sperimentale delle Venezie (Italy) | | | Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health (Germany) | | | WUR Biointeractions & Plant Health (The Netherlands) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Rep. 1 | Rep. 2 | Rep. 3 | Rep. 1 | Rep. 2 | Rep. 3 | Rep. 1 | Rep. 2 | Rep. 3 | Rep. 1 | Rep. 2 | Rep. 3 |
| *A. tumida* | Italy (Cosenza-Calabria) | A | 19.23 | 18.69 | 18.50 | 27.31 | 27.19 | 27.17 | 27.63 | 28.26 | 26.23 | 21.91 | 21.97 | 21.91 |
| *A. tumida* | Burkina Faso (Bobo Dioulasso) | B | 15.43 | 16.27 | 16.63 | 25.11 | 25 | 24.22 | 24.36 | 26.65 | 24.67 | 18.45 | 18.71 | 18.66 |
| *A. tumida* | Tanzania (Arusha) | B | 24.63 | 24.25 | 24.23 | 31.24 | 31.25 | 31.11 | 30.51 | 30.32 | 31.25 | 24.11 | 24.38 | 24.35 |
| *A. tumida* | Philippines (Davao) | C | 27.31 | 26.63 | 26.74 | 32.56 | 32.03 | 32.13 | 33.48 | 33.39 | 33.23 | 26.27 | 26.40 | 26.15 |
| *A. flavicollis* | South Korea | - | 25.26 | 25.59 | 26.15 | 34.82 | 35.06 | 34.83 | 37.6 | 37.6 | 37.08 | 29.19 | 29.20 | 28.99 |
| *A. concolor* | Australia | - | 31.31 | 30.10 | 27.26 | nd | nd | nd | nd | nd | 43.99 | 36.62 | 35.09 | 36.33 |
| *A. mellifera* | Switzerland | - | 18.29 | 18.56 | 18.86 | 25.72 | 27.03 | 25.92 | 27.42 | 27.09 | 27.75 | 22.11 | 22.25 | 22.33 |
| - | Negative control ($H_2O$) | - | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |

**Table 4.** Inter-laboratory sensitivity comparison test of the Li et al. (2018) method for the PCR detection of small hive beetles. Ten-fold dilutions of small hive beetle DNA were screened (blind test) in each laboratory. Positive detection is expressed by the respective Cq value (small hive beetle = SHB; No detection (nd)).

| Sample name | DNA Concentration (ng/µl) | Institute of Bee Health (Switzerland) | | | Istituto Zooprofilattico Sperimentale delle Venezie (Italy) | | | Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health (Germany) | | | WUR Biointeractions & Plant Health (The Netherlands) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Rep. 1 | Rep. 2 | Rep. 3 | Rep. 1 | Rep. 2 | Rep. 3 | Rep. 1 | Rep. 2 | Rep. 3 | Rep. 1 | Rep. 2 | Rep. 3 |
| SHB DNA dilution 1 | 5.00E-03 | 26.32 | 25.29 | 26.12 | 33.13 | 32.39 | 32.44 | 37.27 | 41.99 | 37.67 | 30.98 | 30.76 | 31.06 |
| SHB DNA dilution 2 | 5.00E-04 | 29.52 | 29.62 | 29.79 | 36.38 | 36.29 | 35.58 | nd | 43.55 | 41.11 | 34.64 | 35.08 | 34.47 |
| SHB DNA dilution 3 | 5.00E-05 | 33.82 | 32.35 | 33.17 | nd | 39.25 | nd | nd | nd | nd | nd | 39.21 | 39.04 |
| SHB DNA dilution 4 | 5.00E-06 | 35.01 | 36.36 | 36.08 | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| SHB DNA dilution 5 | 5.00E-07 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| SHB DNA dilution 6 | 5.00E-08 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| SHB DNA dilution 7 | 5.00E-09 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| Non-template control | - | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |

can be used as an alternative more economic method because it does not require labelled probes compared to a qPCR hydrolysis method. From those two methods, the one published by Li et al. (2018) was slightly less variable regarding the detection thresholds (Cq values) and more importantly, having all specimens detected lower than Cq of 25, which is a clear indicator of the robustness of the method (Figs 1, 2).

No false negative results were observed with the Li et al. (2018) and the modified Idrissou et al. (2018) methods. The Li et al. (2018) method was able to confirm the identity of all SHB specimens even on individuals with 2 (Italy: Calabria; Ethiopia: Sidama; Congo: Lume) and 3 (Burkina Faso: Bobo Dioulasso and Tenkodoge) accumulated mismatches. Similarly, the Idrissou et al. (2018) method confirmed the identity of SHB specimens even on individuals with 3 (Uganda: Busiwu), 4 (Burkina Faso: Fada N'gourma) and 5 (Burkina Faso: Bobo Dioulasso) accumulated mismatches.

Regarding the sensitivity for each method, the comparison of the Cq values from the same samples across the methods provides hints for the robustness. A method can be considered more sensitive when the fluorescent amplification curve

crosses the threshold at lower Cq values. Li et al. (2018) detection Cq values range from 13.26 to 24.07, while the modified Idrissou et al. (2018) Cq values range from 10.58 to 26.32 (Figs 1, 3, 4). However, for the Ward et al. (2007) and Silacci et al. (2018) methods, the sensitivity is much lower (Cq values above 30) compared to both Li et al. (2018) and modified Idrissou et al. (2018) methods (Figs 1, 3, 4). Moreover, for the samples with higher variability, which exceed the limits of agreement across the methods (Fig. 2), the Li et al. (2018) method showed to be the most reliable as it detects the SHB samples at lower Cq values.

Regarding the false negative results, they are intrinsically linked to the nucleotide mismatches between the sequences of the primers and probes against the target genome. The Ward et al. (2007) method was unable to detect a specimen from Burkina Faso (Bobo Dioulasso). This specimen accumulated 16 nucleotide mismatches in forward, reverse primers and probe all together (Suppl. material 1: fig. S2). Additionally, several specimens that were at the limit of detection showing high Cq values (Cq > 35) also accumulated several nucleotide mismatches (*i.e.* Burkina Faso: Tenkodoge (15 mutations), Burundi: Rusiga (8 mutations), Burkina Faso: Fada N'gourma and Congo: Lume (7 mutations); Fig. 3; Suppl. material 1: fig. S2). The Silacci et al. (2018) method seems to be more sensitive to mismatches. Depending of the mismatch nucleotide site, a single mutation in the probe is apparently able to produce a false negative *i.e.* Burundi (Rusiga). However, with mismatches at different nucleotide sites, the method was able to detect samples with 2 accumulated mismatches (*i.e.* Portugal and Philippines).

To validate those results, a blind ring test was conducted. Overall, the results matched what was previously observed when all methods were compared ("Comparison of detection methods" section). For example, in the blind ring test, the Wageningen University & Research (WUR) Biointeractions & Plant Health laboratory used the Li et al. (2018) method, with their own modifications in the probe (van Gent-Pelzer and Cornelissen 2021; Suppl. material 1: table S2), and did not produce any false positive or false negative results. In contrast, the results of the laboratories using the Ward et al. (2007) method were consistent with the reporting of the false negatives, which were observed for this particular method. The inter-laboratory comparison using the single chosen method (Li et al. 2018) showed a uniform consistence between all laboratories again without any false positive or false negative results. This result also shows that the method can be adapted to the operating differences in each laboratory (e.g., qPCR reagents, thermocycler types, operators).

The comparison of the various genetic detection methods allowed the evaluation of their strengths and limitations. The error types I and II are of particular importance. The evaluation showed that all methods performed with ideal accuracy regarding error type I, as no false positive was detected even when including several specimens from the genus *Aethina*. In contrast, there were differences in their performances regarding error type II as some SHB specimens were not detected by some methods. The Idrissou et al. (2018) and Li et al. (2018) methods have less sensitivity to nucleotide mismatches on the primer and probe's target sequences. The methods designed more recently performed better as more genetic information, in terms of more SHB specimen sequences, was available for the COI gene. This allowed for the design of primers and probes in regions with lower polymorphism or when certain polymorphic sites could not be avoided, degenerate nucleotides (W:A/T; Y:C/T) were also used (Li et al. 2018).

## Conclusions

The evaluation of the molecular detection methods for SHB, clearly showed that both the Idrissou et al. (2018) and Li et al. (2018) methods avoid both false positives and negatives even when testing across the endemic and introduced regions. However, in view of its higher sensitivity among the tested methods, we propose to recommend the Li et al. (2018) method for the identification of SHB. Global application of such reliable molecular diagnostic tools will contribute to management and control efforts of this mandatory disease and invasive species.

## Acknowledgements

We wish to express our gratitude to the honey bee research association "COLOSS" (https://coloss.org), for providing opportunity for the conception of this project.

## Additional information

### Conflict of interest

The authors have declared that no competing interests exist.

### Ethical statement

No ethical statement was reported.

### Funding

Financial support was granted by the Vinetum Foundation (P.N.).

### Author contributions

Conceptualization: MGP, OY, PN. Data curation: OY. Formal analysis: OY. Funding acquisition: PN. Investigation: OY, AG, MOS, MGP. Methodology: MOS, AG, OY, MGP. Resources: PN. Writing - original draft: PN, OY. Writing - review and editing: PN, AG, MOS, OY, MGP.

### Author ORCIDs

Orlando Yañez https://orcid.org/0000-0001-8493-2726
Marga van Gent-Pelzer https://orcid.org/0000-0002-1880-4344
Anna Granato https://orcid.org/0000-0002-1595-4347
Marc Oliver Schäfer https://orcid.org/0000-0002-9789-1019
Peter Neumann https://orcid.org/0000-0001-5163-5215

### Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

## References

Altman DG, Bland JM (1983) Measurement in Medicine: The Analysis of Method Comparison Studies. Journal of the Royal Statistical Society: Series D (The Statistician) 32: 307–317. https://doi.org/10.2307/2987937

Ellis JD, Hepburn HR (2006) An ecological digest of the small hive beetle (*Aethina tumida*), a symbiont in honey bee colonies (*Apis mellifera*). Insectes Sociaux 53: 8–19. https://doi.org/10.1007/s00040-005-0851-8

Hood WM (2000) Overview of the small hive beetle, *Aethina tumida*, in North America. Bee World 81: 129–137. https://doi.org/10.1080/0005772X.2000.11099483

Idrissou FO, Huang Q, Yañez O, Akinwande KL, Neumann P (2018) PCR Diagnosis of Small Hive Beetles. Insects 9: 24. https://doi.org/10.3390/insects9010024

Idrissou FO, Huang Q, Yañez O, Neumann P (2019) International beeswax trade facilitates small hive beetle invasions. Scientific Reports 9: 10665. https://doi.org/10.1038/s41598-019-47107-6

Li D, Waite DW, Fan Q-H, George S, Semeraro L, Blacket MJ (2018) Molecular detection of small hive beetle *Aethina tumida* Murray (Coleoptera: Nitidulidae): DNA barcoding and development of a real-time PCR assay. Scientific Reports 8: 9623. https://doi.org/10.1038/s41598-018-27603-x

Liu Y, Han W, Gao J, Su S, Beaurepaire A, Yañez O, Neumann P (2021) Out of Africa: novel source of small hive beetles infesting Eastern and Western honey bee colonies in China. Journal of Apicultural Research 60: 108–110. https://doi.org/10.1080/00218839.2020.1816686.

Neumann P, Elzen PJ (2004) The biology of the small hive beetle (*Aethina tumida*, Coleoptera: Nitidulidae): Gaps in our knowledge of an invasive species. Apidologie 35: 229–247. https://doi.org/10.1051/apido:2004010

Neumann P, Pettis JS, Schäfer MO (2016) *Quo vadis Aethina tumida*? Biology and control of small hive beetles. Apidologie 47: 427–466. https://doi.org/10.1007/s13592-016-0426-x.

Schäfer MO, Cardaio I, Cilia G, Cornelissen B, Crailsheim K, Formato G, Lawrence AK, Le Conte Y, Mutinelli F, Nanetti A, Rivera-Gomis J, Teepe A, Neumann P (2019) How to slow the global spread of small hive beetles, *Aethina tumida*. Biological Invasions 21: 1451–1459. https://doi.org/10.1007/s10530-019-01917-x

Silacci P, Biolley C, Jud C, Charrière J-D, Dainat B (2018) An improved DNA method to unambiguously detect small hive beetle *Aethina tumida*, an invasive pest of honeybee colonies. Pest Management Science 74: 2667–2670. https://doi.org/10.1002/ps.5141

van Gent-Pelzer M, Cornelissen B (2021) Detection of small hive beetle: frass as a source of DNA. Journal of Apicultural Research 60: 683–685. https://doi.org/10.1080/00218839.2021.1914964

Ward L, Brown M, Neumann P, Wilkins S, Pettis J, Boonham N (2007) A DNA method for screening hive debris for the presence of small hive beetle (*Aethina tumida*). Apidologie 38: 272–280. https://doi.org/10.1051/apido:2007004

## Supplementary material 1

### Reliable molecular detection of small hive beetles

Authors: Orlando Yañez, Marga van Gent-Pelzer, Anna Granato, Marc Oliver Schäfer, Peter Neumann

Data type: docx

Explanation note: Country of origen of specimens, primers and probes, PCR protocols, melting curve analysis, nucleotide mismatches

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/neobiota.95.124673.suppl1